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Enhancing the Quality and Nutrient Content of Soybean Milk Waste as Poultry Feed Through Fermentation with *Bacillus subtilis*

Gita Ciptaan[1*], Mirnawati[1], Imana Martaguri [1], Kadran Fajrona[1] and Anifah Srifani[2]

[1]Department of Animal Feed and Nutrition, Faculty of Animal Science, Andalas University, Padang 25163, Indonesia.
[2]Doctoral student, Department of Animal Feed and Nutrition, Faculty of Animal Science, Andalas University,Padang, 25163, West Sumatra, Indonesia.
***Corresponding author:** gciptaan@ansci.unand.ac.id

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ABSTRACT

This study was conducted to understand the effects of substrate composition and length of fermentation for improving the content and quality of soybean milk waste as poultry feed. The materials used in this study were soybean milk waste (SMW), rice bran, Indigofera leaf flour (ILF), and *Bacillus subtilis.* A completely randomized design (CRD) was used with a factorial pattern (3 x 3) and 3 replications. Factor A was the composition of the substrate, (A1) 100% SMW, (A2) 80% SMW + 20% rice bran, and (C) 80% SMW + 20% ILF. Factor B was the length of fermentation that consisted of (B1) 2 days, (B2) 4 days, (B3), and 6 days. Parameters measured were phytase activity (PA), crude protein (CP), nitrogen retention (NR), crude fiber (CF), crude fiber digestibility (CFD), and metabolic energy (ME) of SMW fermented with *B. Subtilis*. There was an interaction ($P<0.05$) between substrate composition and fermentation time of SMW fermented with *Bacillus subtilis* on phytase enzyme activity, crude protein, and nitrogen retention. Substrate composition of 80% SMW + 20% ILF and 6 days of fermentation time was the best result which was able to increase phytase enzyme activity (6.71U/mL), crude protein content (41.82%), and nitrogen retention (61.41%) of fermented soybean milk waste. There was an alleviate in crude fiber content (10.49%), an increase in crude fiber digestibility (57.29%), and metabolic energy (2199.80kcal/kg) of SMW fermented with *B subtilis.*

Key words: Substrate composition, Fermentation time, Soybean milk waste, *Bacillus subtilis*, Phytase

INTRODUCTION

Soybean milk waste (SMW) contains nutrients, namely 24.76% crude protein, 2.86% crude fat, 18.15% crude fiber, 0.087% Ca, 0.053% P, 3915.95 Kcal/kg gross energy, 2.98% phytic acid (Ciptaan et al. 2018). SMW had potential as an alternative feed ingredient, but the high phytic acid in SMW causes SMW to be used only 6.2% in broiler rations (Mirnawati et al. 2012). The high content of crude fiber and phytic acid is the reason for the low usage of SMW in broiler ration because poultry cannot digest these two nutrients.

Poultry had limited cellulase enzymes in their digestive tract (Ojha et al. 2019; Tüzün et al. 2020; Haribhau et al. 2020; Haque et al. 2021) so it made them unable to digest high crude fiber. Poultry is also unable to digest phytic acid due to the absence of phytase enzymes in their digestive tract (Abbasi et al. 2019). Phytic acid in a large amount will suppress the digestibility of nutrients like protein (Samtiya et al. 2020). Phytic acid cannot be hydrolyzed in the digestive tract of monogastric animals because phytic acid could make insoluble complex compounds with salt that prevent the absorption of minerals, whereas mineral deficiency in the body can cause metabolic disorders (Sari 2012; Erdaw and Beyene 2018).

To diminish crude fiber and phytic acid in SMW, it was necessary to carry out fermentation using cellulolytic and phytolytic microbes. Enzymes produced by microbes during fermentation can enforce metabolic processes such as oxidation, reduction, hydrolysis, and other chemical reactions, resulting in chemical changes occurring in an organic substrate to produce certain products (Prescott et al. 2004). One of the microbes that are cellulolytic and phytolytic is *Aspergillus ficuum* (Aini et al. 2023).

Ciptaan et al. (2022) carried out SMW fermented with a combination of *A. ficuum and Neurospora crassa* (3:2) inoculums with 7 days' fermentation, and got a crude protein content of 28.25%, 13.77% crude fiber, nitrogen retention 61.16 %, 1.15% crude fat, 0.11g/100g phytic acid, 4012 µg/100g carotenoids. This feed ingredient had been

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tried to replace commercial feeds up to 25% in broiler rations. Ciptaan et al. (2018) also did SMW fermentation with 10% *A. ficcum* for 9 days and found advanced results seen from crude protein content of 34.95%, nitrogen retention of 62.99%, protease activity of 7.76U/mL, phytase activity 7.49U/mL, cellulase activity was 48.55U/mL, crude fiber content was 11.01%, crude fiber digestibility was 58.92%, and phytic acid was 0.11%, carotenoid 4012µg/100g. SMW fermented with *A. ficuum* and *N. crassa* as inoculums can be used to 25% in broiler ration (Ciptaan et al. 2022).

The problem with fermentation using *A. ficuum* is the long fermentation time (7-9 days). Therefore, it is necessary to use other cellulolytic and phytolytic microorganisms with a faster incubation time. One of the bacteria that can be used is *Bacillus subtilis* (Azam et al. 2023). This bacterium is a phytolytic bacterium with the highest phytase activity, namely 378U/mL (Sing et al. 2013), and the highest protease activity, namely 2.162U/mg with 6 hours of the incubation period (Efendi et al. 2017). *Bacillus subtilis* is also a probiotic, so the addition of *B. subtilis* probiotics until 500g/ton ration can maintain carcass and meat production for male broilers with lower feed intakes (Hananto 2014). Mirnawati et al. (2019) conducted a study on palm kernel cake (PKC) fermentation with a combination of inoculum dose and fermentation time using *B. subtilis*, it was found that a 7% inoculum dose of *B. subtilis* with 6 days of fermentation was the best combination which resulted in mannanase activity of 24.27U/mL, protease activity of 10.27U/mL, and cellulase activity 17.13U/mL. It is expected that SMW fermentation with *B. subtilis* can enhance SMW quality so that it can be used in poultry rations.

In fermentation, several factors need to be considered, namely the composition of the substrate. Nutrients contained in the substrate are needed by microbes to live, increase body mass, and affect the enzymes produced by microbes. The mixtures of substrates used in this study were SMW with rice bran and SMW with Indigofera Leaf Flour (ILF). According to Akbarillah et al. (2010), ILF contains quite high pigmentations such as xanthophylls and carotenoids. It is expected that one of these substrate mixtures can increase the content and quality of SMW after fermentation.

Rice bran contains 13.5% crude protein, 13% crude fiber, 10.66% crude fat, 53.69% BETN, 4.81% lysine, and 2.32% methionine (Rasyaf 2002). While the nutritional contents of Indigofera leaves are 27.97% crude protein, 15.25% crude fiber, 0.22% Ca, 0.18% phosphorus, and 507.6 mg/kg β-carotene content (Palupi et al. 2014).

The addition of rice bran to the substrate is a common practice, although the protein content is low, the rice bran has a loose nature that makes it easier for microorganisms to grow properly. On the other hand, Indigofera leaves contain quite a high amount of protein and β-carotene. It is expected that one of these substrate mixtures can increase the content and quality of SMW after fermentation. Another factor that also affects fermentation is the time of fermentation (Choi et al. 2019). Time determines the amount of enzymes produced in fermentation. Fermentation time generally depends on the type of organism and the substrate used (Pasaribu 2007). More microbes will grow and more enzymes will be produced in a long time of fermentation (Mirnawati et al. 2019). This study was conducted to understand the effects of substrate composition and length of fermentation for improving the content and quality of soybean milk waste as poultry feed.

MATERIALS AND METHODS

Ethical Approval

The present study was approved by the Animal Ethics Committee of Andalas University, West Sumatera, Indonesia.

Research Design

This study used a completely randomized design (CRD) 3x3 factorial with 3 replications. Factor A was the composition of the substrate, consisting of A1=100% SMW, A2=80% SMW + 20% Rice Bran, and A3=80% SMW + 20% ILF. Factor B was the length of fermentation, namely: B1=2 days, B2=4 days, and B3=6 days.

Research Implementation

Sample Preparation

The soybean milk waste material was taken from a soymilk manufacturing business located in Lohong Village, East Pariaman District, Pariaman City, West Sumatra, Indonesia. Samples of soybean milk waste were dried in the oven at 50-60℃ for 24-48 hours or dried in the sun until they remained at a constant weight (as fed). After drying, the samples were milled until smooth. The Indigofera leaf flour (ILF) used were finished ingredients that were ready to use.

Bacterial Rejuvenation

Bacterial rejuvenation was carried out using NA (nutrient agar) media. NA and distilled water were cooked until clear, it was put into the test tube and petri dish. It was then sterilized in the autoclave at 121ºC for 15min. Pure inoculation of *B. subtilis* was then cultured using a sterile loop for 24 hours at room temperature. The total colonies were observed and counted after incubation for 24 hours.

Making Inoculums of *Bacillus subtilis*

The preparation of *Bacillus subtilis* inoculum was carried out using a substrate, 100g of rice bran, added 70mL distilled water, and then sterilized in an autoclave for 15min at 121ºC at 1 atm pressure. It was then placed at room temperature (24ºC). After that, the bacteria were diluted in a test tube with 7mL of Brook solution and inoculated on the rice bran substrate. It was then incubated for 4 days. After incubation, the inoculum was ready for use in fresh form.

Substrate Preparation

The substrates used were soybean milk waste (SMW), rice bran and Indigofera leaf flour (ILF) according to the treatments A1 (100% SMW), A2 (80% SMW: 20% rice bran), A3 (80% SMW:20% ILF). Each treatment was weighed as much as 100g according to its composition (A1 = 100g SMW, A2 = 80g SMW + 20g rice bran, A3 =80g SMW + 20g ILF) and put into polypropylene plastic measuring 15x25 cm. About 70mL of distilled water was added to it and sterilized in the autoclave for 15min at

121℃ with a pressure of 1 atm. After the substrate was sterilized, the then fermentation was carried out.

Fermentation Implementation

The sterilized substrate was inoculated with 10% *B. subtilis* inoculum (Mirnawati et al. 2019) and then incubated according to the fermentation duration treatment of B1 (2 days), B2 (4 days), B3 (6 days). After being fermented, all of the materials were harvested and dried in an oven at 50-60℃ until the weight remained constant, then milled and analyzed for nutritional content according to parameters.

Phytase Enzyme Activity

Measurement of phytase activity was carried out according to Kim and Lei (2005). Bacterial isolates were grown in MRS broth for 24 hours at 37ºC, then centrifuged at 13,000 rpm for 5 minutes. A total of 0.2 mL of the enzyme supernatant was added to 0.2 mL of phytic acid in acetate buffer pH 5.5. After that, it was incubated at 37ºC for 15 minutes and then the reaction was stopped by adding 0.4 mL of 10% TCA. After that, it was centrifuged at 2000 rpm for 15 minutes. The supernatant was transferred into a new test tube. Then 2 mL of reagent (1 M sulfuric acid and 2.5% ammonium molybdate and 10% ascorbic acid) were added in a ratio of 3:1:1 and homogenized. The mixture was then incubated at 50°C for 15 minutes, cooled, and then the absorbance of each sample was measured. at 420 nm. Enzyme activity was expressed in U/mL, which was µmol PO4-3 released per minute per milliliter of the enzyme.

Crude protein was estimated by using Kjeldahl's method (1883). Crude fiber, nitrogen retention, crude fiber digestibility, and metabolic energy were measured using Sibbald's method (1975).

Data Analysis

All data obtained were analyzed using analysis of variance (ANOVA) through statistical analysis systems. The Duncans Multiple Range Test (DMRT) test was used to determine differences between treatments (Steel and Torrie 2002).

RESULTS AND DISCUSSION

The average phytase activity (PA), crude protein (CP), nitrogen retention (NR), crude fiber (CF), crude fiber digestibility (CFD), and metabolic energy (ME) of soymilk waste fermentation (SMWF) with *B. subtilis* can be seen in Tables below.

Phytase Activity (PA)

Data from the variant analysis indicated a very significant interaction ($P<0.01$) between factor A (substrate composition) and factor B (fermentation time), while each factor of A and B also had a very significantly different effect ($P<0.01$) on crude fiber content (Table 1). From this result, it can be concluded that the longer the time given, the higher the phytase activity in treatments A1, A2, and A3. Likewise, phytase activity in the substrate composition with the addition of 20% ILF gave higher results in B1, B2, and B3 treatments. The optimal phytase activity was in the A3B3 treatment.

High PA was found in the A3B3 treatment (80% SMW + 20% ILF) because ILF had better nutrition so more microbes grew in the treatment. The protein contained in ILF is utilized for the formation of microbes in the substrate. That was per the findings of Seftiadi et al. (2020) that microbes work according to the inducers available on the substrate. The high activity of the phytase enzyme in A3B3 was also due to the longer fermentation time which was 6 days. A longer fermentation proceeds to the ding's growth of many microbes, thus more enzymes were going to be produced. In addition, a longer fermentation will give opportunities for microbes to degrade more substrates and it could increase the nutritional content of substrates (Lee et al. 2019). This was in line with Mirnawati et al. (2019a) who stated that at the beginning of fermentation, the enzyme activity was still low because there was little microbial growth and would be increasing as the increase of fermentation time. It is known from the results that the A3B3 gave PA of 6.71U/mL. This result is lower than the result obtained by Ciptaan et al. (2018) where fermentation of SMW with 10% *A. ficuum* for 9 days obtained PA of 7.49U/mL.

Crude Protein (CP)

The average CP content of SMWF with *B. subtilis* is shown in Table 2. Data of variance analysis indicated a very significant interaction ($P<0.01$) between the factors, while each factor of A and B had a highly significant ($P<0.01$) effect on the CP content. From this study, it was known that the longer the fermentation time, the higher the CP content of SMWF in treatments A1, A2 and A3. Likewise, CP in the substrate composition with the addition of 20% ILF gave higher content in both B1, B2 and B3 treatments. The optimal CP is found in the A3B3 treatment.

The high CP in the A3B3 treatment (80% SMW + 20% ILF) was caused by more microbes growing due to the addition of 20% ILF. Indigofera leaf flour has better nutrition which can help microbes to grow and develop. In addition, the higher CP content of the substrate before fermentation results in the higher CP content obtained after. In addition, the rice bran which was mixed as a substrate also has loose properties making it easier for microorganisms to grow properly. The work of bacteria in secreting enzymes is influenced by their inducers (Dobretsov and Rittschof 2020). The higher the protein content of inducers, the efforts of bacteria to secrete protease enzymes into the environment to decompose proteins into amino acids will be higher (Oetari 2006).

From the above results, it is known that the A3B3 treatment provided a CP content of 41.82%. This result was higher than the result obtained by Ciptaan et al. (2022) where fermented soymilk waste with *A. ficuum* and *N. crassa* (3:2) for 7 days provided protein about 28.25%.

Nitrogen Retention (NR)

Data in Table 3 shows that there was a highly significant ($P<0.05$) different interaction between the factors, while each factor of A and B also had a highly significant ($P<0.01$) effect on NR content. From the data (Table 3), it can be said that the longer the time given, the higher the SMWF of NR both in treatments A1, A2, and A3. Likewise, NR in the substrate composition with the addition of 20% ILF gave higher yields in both B1, B2 and B3 treatments. Optimal NR is A3B3 treatment.

Table 1: The average content of phytase activity of SMWF with *B. subtilis*

Parameters	Factor A (substrate composition)	Factor B (length fermentation)			Average	SEM	p
		B1 (2 days)	B2 (4 days)	B3 (6 days)			
Phytase activity (U/mL)	A1 (100% SMW)	2.13cC	3.04bC	4.51aC	3.23	0.35	P<0.05
	A2 (80% SMW+20% Rice Bran)	3.30cB	4.73bB	5.66aB	4.56	0.35	p<0.05
	A3 (80% SMW+20% ILF)	4.13cA	5.93bA	6.71aA	5.59	0.38	p<0.05

Note: Different lowercase and uppercase letters in the same row and column have a highly significant different effect (P<0.01). Treatment interaction had no significant effect (P>0.05).

Table 2: The average content of crude protein of SMWF with *B. subtilis*

Parameters	Factor A (substrate composition)	Factor B (length fermentation)			Average	SEM	p
		B1 (2 days)	B2 (4 days)	B3 (6 days)			
Crude Protein (%)	A1 (100% SMW)	29.40cC	32.67bC	35.60aC	32.56	0.90	p<0.05
	A2 (80% SMW+20% Rice Bran)	31.40cB	35.13bB			1.08	p<0.05
	A3 (80% SMW+20% ILF)	33.57cA	36.87bA	41.82aA	37.42	1.20	p<0.05

Note: Different lowercase and uppercase letters in the same row and column have a highly significant different effect (P<0.01). Treatment interaction had no significant effect (P>0.05).

Table 3: The average content of nitrogen retention of SMWF with *B. subtilis*

Parameters	Factor A (substrate composition)	Factor B (length fermentation)			Average	SEM	p
		B1 (2 days)	B2 (4 days)	B3 (6 days)			
Nitrogen Retention (%)	A1 (100% SMW)	49.12cA	54.01bA	58.37aA	53.83	1.35	P<0.05
	A2 (80% SMW+20% Rice Bran)	51.29cB	55.16bB	59.68aB	55.38	1.26	P<0.05
	A3 (80% SMW+20% ILF)	52.75cC	57.91bC	61.41aC	57.36	1.26	P<0.05

Note: Different lowercase and uppercase letters in the same row and column have a highly significant different effect (P<0.01). Treatment interaction had no significant effect (P>0.05).

High NR found in A3B3 (80% SMW + 20% ILF) was caused by high protein content in that treatment. McDonald et al. (2002) reported that protein content in the material affects the percentage of NR. High protein content will cause an increase in NR. The level of nitrogen in the excreta affects the amount of NR. The high content of nitrogen in the body would make less nitrogen wasted in excreta (Maynard et al. 2005). In addition, the level of NR is influenced by the phytase produced in fermentation. This was per the findings of Shelton et al. (2004) that phytase enzyme can hydrolyze phytate bonds so it increases the protein availability, digestible amino acid, and NR content.

From the above results, it is known that the A3B3 treatment provided an NR content of 61.41%. This result is higher than the result obtained by Ciptaan et al. (2022) where fermentation of soymilk waste with *A. ficuum* and *N. crassa* (ratio 3:2) for 7 days gave nitrogen retention of 61.16%.

Crude Fiber (CF)

A very significant interaction (P<0.01) was found between the factors (A & B), whereas each factor of A and B also had a highly significant (P<0.01) effect on the CF content (Table 4). It can be seen from the data that the longer fermentation, the lower the CF content of SMWF in A1, A2, and A3 treatments. Likewise, CF content in substrate composition with the addition of 20% ILF showed lower results in treatments B1, B2, and B3. The lowest CF was found in the A3B3 treatment.

The alleviation of CF content in A3B3 (80% SMW + 20% ILF) with a 6-day fermentation was caused by the large number of bacteria that grow and develop. The more bacteria grow, the more enzymes are produced, especially the cellulase enzyme which can help to reduce the crude fiber content (Islam and Roy 2018). This result was following the findings of Sudarmono et al. (2016) that the more growth in microbes, the more cellulase will be produced to alter cellulose into simple sugars which causes the decrease of CF content after fermentation. A3B3 treatment with a fermentation time of 6 days gave the lowest crude fiber content (10.39%).

The treatment of A3B3 treatment provided the best crude fiber reduction compared to other treatments. The results obtained are lower than the results obtained by Ciptaan et al. (2018) where fermentation of SMW with 10%. *A. ficuum* for 9 days resulted in a crude fiber content of 11.01%.

Crude Fiber Digestibility (CFD)

A very significant interaction (P<0.01) was found between the factors (A&B) while each factor of A and B also had a very significantly different effect (P<0.01) on CFD. The longer fermentation time further increased DCF in A1, A2, and A3 treatments. Likewise, CFD in the substrate composition with the addition of 20% Indigofera leaf meal gave higher yields in both treatments B1, B2, and B3. The highest CFD was found in A3B3.

An increase of CFD was found in A3B3 (80% SMW + 20% ILF) with a fermentation time of 6 days due to the low content of CF. The lower CF gave a result in the higher CFD. The result was in concur with Mirnawati et al. (2017) that the CFD relies on the crude fiber content of the feed, high content of CF would be lowering the digestibility of crude fiber due to the limitations of poultry to digest crude fiber. From the above data, it can also be concluded that A3B3 with 6 days of fermentation provided the highest increase in crude fiber digestibility, namely 56.51%.

From the above results (Table 5), it was known that the A3B3 treatment increased CFD. This result was lower than the result obtained by Ciptaan et al. (2022) where fermentation of SMW with *A.s ficuum and N. crassa* (3:2) for 7 days increased the digestibility of crude fiber by 58.76%.

Table 4: The average content of crude fiber of SMWF with *B. subtilis*

Parameters	Factor A (substrate composition)	Factor B (length fermentation)			Average	SEM	p
		B1 (2 days)	B2 (4 days)	B3 (6 days)			
Crude Fiber (%)	A1 (100% SMW)	16.61aA	15.66bA	14.72cA	15.66	0.54	p<0.05
	A2 (80% SMW+20% Rice Bran)	15.76aB	14.30bB	12.42cB	14.16	0.97	p<0.05
	A3 (80% SMW+20% ILF)	13.61aC	11.79bC	10.39cC	11.93	0.93	p<0.05

Note: Different lowercase and uppercase letters in the same row and column have a highly significant different effect (P<0.01). Treatment interaction had no significant effect (P>0.05).

Table 5: The average content of crude fiber digestibility of SMWF with *B. subtilis*

Parameters	Factor A (substrate composition)	Factor B (length fermentation)			Average	SEM	p
		B1 (2 days)	B2 (4 days)	B3 (6 days)			
Crude Fiber Digestibility (%)	A1 (100% SMW)	45.65cC	46.56bC	48.77aC	46.99	0.93	p<0.05
	A2 (80% SMW+20% Rice Bran)	46.82cB	48.28bB	51.62aB	48.91	1.42	p<0.05
	A3 (80% SMW+20% ILF)	50.54cA	54.40bA	56.51aA	53.82	1.75	p<0.05

Note: Different lowercase and uppercase letters in the same row and column have a highly significant different effect (P<0.01). Treatment interaction had no significant effect (P>0.05).

Table 6: The average content of metabolic energy of SMWF with *B. subtilis*

Parameters	Factor A (substrate composition)	Factor B (length fermentation)			Average	SEM	p
		B1 (2 days)	B2 (4 days)	B3 (6 days)			
Energy metabolism (kcal/kg)	A1 (100% SMW)	1273.53cA	1310.26bA	1565.13aA	1382.97	91.69	P<0.05
	A2 (80% SMW+20% Rice Bran)	1317.58cB	1414.70bB	1934.44aB	1555.57	191.50	P<0.05
	A3 (80% SMW+20% ILF)	1878.81cC	2167.38bC	2403.22aC	2149.80	151.64	P<0.05

Note: Different lowercase and uppercase letters in the same row and column have a highly significant different effect (P<0.01). Treatment interaction had no significant effect (P>0.05).

Metabolizable Energy (ME)

A very significantly different interaction (P>0.05) was found between the factors, whereas each factor of A and B also had a very significantly different effect (P<0.01) on ME (Table 6). The longer the time given, the higher the energy content of SMWF metabolism in treatments A1, A2, and A3. Likewise, the ME of the substrate composition with the addition of 20% ILF gave higher yields in both B1, B2, and B3 treatments. The highest metabolizable energy was in the A3B3 treatment.

An increase in the ME content of A3B3 (80% SMW + 20% ILF) was associated with a decrease in CF content and an increase in CFD, causing an increase in the energy utilized by livestock. Sekh and Karki (2022) stated that the high fiber content of feed reflects lower metabolizable energy (ME). This can be due to the limitation of the broiler to digest CF. From the above results, it is known that the A3B3 treatment provided an increase in metabolizable energy of 2403.22 kcal/kg. The result found in this study was lower than the result from Mirnawati et al. (2012) where SMWF with *N. crassa* used a substrate of 70% SMW + 30% rice bran) with a metabolizable energy content of 2767 kcal/kg.

Conclusion

Based on this study, it can be deduced that there was an interaction between the composition of the substrate and the fermentation time of SMW with *Bacillus subtilis*. Substrate composition of 80% SMW + 20% ILF with 6 days of fermentation time was the best result which was able to increase phytase enzyme activity (6.71 U/mL), crude protein content (41.82%), nitrogen retention (61.41%), crude fiber (10.39%), digestibility of crude fiber (56.51%), and metabolizable energy (2199.80 kcal/kg) of fermented SMW.

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Author's Contribution

Gita Ciptaan was in charge to supervise the experiment and writing the original script. Mirnawati, Imana Martaguri and Kadran Fajrona conducted the experiment and analyzed the data. Anifah Srifani finalized the manuscript.

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